1966

Parathyroid Extract-Induced Alterations in the Oxidation of Organic Acids in Bone

Allyn F. DeLong
Loyola University Chicago

PARATHYROID EXTRACT-INDUCED ALTERATIONS IN THE OXIDATION OF ORGANIC ACIDS IN BONE

by

Allyn F. DeLong

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of

Master of Science

June

1966

LIFE

Allyn F. DeLong was born in Reading, Pennsylvania on April 12, 1942.

He was graduated from Pottstown High School, Pottstown, Pennsylvania in June 1960, and received a Bachelor of Science degree from Ursinus College, Collegeville, Pennsylvania in June 1964.

In September 1964, he began his graduate studies in the Department of Biochemistry and Biophysics, Loyola University, Stritch School of Medicine. From September 1964 to June 1965, he served as a graduate teaching assistant for the department. He has been a National Institute of Health Trainee since July 1965.

On December 26, 1965, he married Gloria F. Renninger.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Maurice V. L'Heureux for his guidance in the preparation of this thesis and the research contained therein and to the other staff members and fellow graduate students who have contributed to the author's work by exchange of ideas.

The author also wishes to thank Dr. A. S. Ridolfo, the Lilly Laboratory for Clinical Research, Indianapolis, Indiana, for making available the parathyroid extract used for this research.

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TABLE OF ABBREVIATIONS

EDTA - Ethylenediaminetetracetic Acid

NAD - Nicotinamide Adenine Dinucleotide

NADP - Nicotinamide Adenine Dinucleotide Phosphate

PTE - Parathyroid Extract

PTH - Parathyroid Hormone

PTX - Parathyroidectomized

S.A. - Specific Activity

CHAPTER I

INTRODUCTION

Hanson (25) and Collip (13) independently accomplished the successful isolation of a physiologically active extract of the parathyroid glands. The administration of the preparation to thyroparathyroidectomized dogs consistently prevented or relieved the symptoms of tetany. Recently, workers in the field have obtained highly purified preparations of parathyroid hormone. Aurbach (5), using Sephadex G-100 columns, recovered a hormone preparation which was better than 90% pure polypeptide having a molecular weight of 10,000. Rasmussen (27, 50) has isolated from bovine parathyroid glands three polypeptides which have varying amounts of physiological activity. One peptide affects calcium mobilization and phosphate excretion, but has no affect on glycolysis. The second and third peptides affect glycolysis to varying degrees, but do not possess the hypercalcemic and phosphaturic responses of the first.

In 1908, MacCallum and Voegtlin (36) discovered the relation of the nervous manifestations (tetany) of parathyroidectomy to the levels of serum calcium. This was the beginning of the study of the relationship between the parathyroid glands and plasma calcium levels.

It is generally accepted that the parathyroid glands monitor the calcium ion concentration in blood mainly by

influencing the mobilization of calcium from the skeleton. The parathyroid hormone also exerts influence on the excretion of phosphate by the kidneys. While the primary effect of the parathyroid hormone is upon the mobilization of calcium from bone, there is no evidence for the influence in mineral deposition in the organic matrix.

The homeostasis of calcium constitutes a special case among the cations of the blood plasma. Homeostatic control of the concentrations of other cations of the fluids of the body, and their retention by the kidneys is usually accomplished by integration of neural and hormonal influences. The parathyroid glands themselves act as receivers of information directly from the calcium ion concentration in the plasma and respond by altering endogenous levels of hormone secretion which will correct any deviation from normal. Patt and Luckhardt (49) demonstrated by perfusion of the parathyroid glands with serum depleted of calcium, that these glands respond directly to a lowered concentration of calcium in the plasma by increased secretory activity. Current evidence also supports this view. (14, 22, 38, 55). Patt and Luckhardt further demonstrated that when the perfusate was injected intravenously into normal dogs, there was an increase in serum calcium within one and one-half to three hours and in most cases an increase in serum inorganic phosphate of almost the same magnitude.

Many studies on the effect of parathyroid hormone on the

kidney have been reported (1, 2, 15, 20, 24, 30, 34). As early as 1952, Greenwald (23) showed a reduced urinary excretion of phosphate following parathyroidectomy. Recent evidence confirms this idea (2, 6, 8, 52). Albright and Ellsworth (1, 48) first demonstrated the phosphaturic action of parathyroid hormone on a patient with hypoparathyroidism. Ito et al. (31) found a 70% increase in the tubular reabsorption of phosphate following parathyroidectomy. Samiy (51) found that parathyroid hormone injected into parathyroidectomized dogs increased the phosphate excretion but decreased the tubular reabsorption. The effects appeared to be directly on the reabsorption in both proximal and distal segments of the nephron.

Most research in this field indicates that parathyroid hormone induces a net decrease in the reabsorption of phosphate in the proximal tubules of the kidney. However, the possibility of an induced active secretion of phosphate in the distal tubules of the kidney cannot be ruled out as an alternative explanation. Phosphate secretion has been shown to occur in various mammals. Levinsky and Davidson (34) have shown both phosphate secretion in the kidney and an effect of parathyroid hormone upon this secretion in the chicken. Brodsky (7) has interperted his data to mean that there is a secretion of phosphate in dogs. Nicholson (46, 47) damaged proximal and distal tubules in dogs by the use of nephrotoxic agents. Proximal

tubular damage caused increased phosphate excretion, and distal tubular damage caused a decreased phosphate excretion. Parathyroid hormone-induced phosphate diuresis was prevented by distal tubular damage, but was not prevented by proximal tubular damage. The results of the experiment with normal dogs were interperted to mean that the distal tubules in the dog kidney are the active site of secretion of phosphate. The parathyroid hormone-induced phosphate diuresis appears to be the result of increased secretion of phosphate in the distal tubles.

Bone is the chief target organ of parathyroid hormone in the regulation of blood calcium. However, it has been demonstrated that parathyroid function is not entirely responsible for blood calcium homeostasis. Talmage and Elliott (53,54) by applying the technique of peritoneal lavage, demonstrated that parathyroidectomized rats could mobilize calcium from bone at the same rate as normal rats. They concluded that the site of action of parathyroid hormone must be on the deeper trabeculae and not on the most recently deposited calcium. It is generally accepted by workers in this field that the parathyroid control mechanism is a slow acting one responsible for the hour to hour calcium homeostasis, and that ion transfer between blood and bone is responsible for the minute to minute homeostasis. Parathyroid function affects the "stable" calcium located in the mineralized areas of bone, while the blood-bone

ion transfer affects the "labile" calcium which occurs in newly forming and surface areas of bone.

A number of observations have been reported which suggest that citric acid plays an important role in the resorption of calcium from the skeleton (26, 44, 39, 40, 48) and that the mechanism of action of parathyroid hormone may be explained by means of some effect on citric acid metabolism. The citric acid cycle has been shown to be present and functioning in bone (18, 32, 53, 59). It has been demonstrated that the injection of parathyroid extract into experimental animals produces an elevation of both serum calcium and citrate (3, 35). The skeleton must be the source of extracellular calcium since the largest proportion of body calcium is found in the bone crystal apatite structure, and dissolution of bone can be observed in response to parathyroid extract. Bone contains large quantities of citrate relative to other tissues. Citric acid is a short-lived substance which is synthesized and metabolized by most tissues.

Neuman et al. (45) have explored the possibility that the increase in plasma citric acid which follows the injection of parathyroid extract is the result of metabolic processes in bone. By drilling a hole in the spongiosa of the femur of an intact dog, the blood was collected from the spongiosal circulation and was compared with that of arterial samples. Increased citrate output from bone of over 1 mg per 100 ml of blood was observed within a few hours after injecting parathyroid extract

but arterial modifications were not observed until much later. Additional studies by Martin et al. (37) have shown that dogs respond to parathyroid extract with net citrate production by bone but not by other tissues.

A much earlier indication of an influence of parathyroid hormone on citric acid metabolism was presented by Dickens in 1941 (17). He reported that the dried alcohol-extracted femur of a normal pup contained 1.13% citric acid whereas the bone from a parathyroid extract-treated pup contained 1.66%. Lekan, Laskin and Engel (33) have confirmed the effect of parathyroid extract on bone citrate. They investigated the formation of radioactive citrate from pyruvate-2-^{14}C by bone slices and reported an increased radioactivity of citrate in bone obtained from parathyroid extract-treated rabbits. Earlier studies of Laskin and Engel (32) indicated the reduced respiration of rabbit femur which was caused by the administration of parathyroid extract. The investigations of Freeman and Chang (21) and Elliott and Freeman (19) provide further data for the relationships between citric acid and parathyroid hormone. They show that a rise in citric acid in blood that accompanies nephrectomy is abolished by prior parathyroidectomy.

In vitro studies by Vaes and Nichols (56) indicate that the metabolism of citrate in mouse metaphysis occurs at about ten times the rate of synthesis. Since bone cells have the capacity to metabolize the acid more rapidly than it can be

formed, it would be more likely that the parathyroid extract effect in bone is on metabolism rather than synthesis. Bone cells can synthesize citric acid, but the elevated levels of citrate obtained upon parathyroid extract administration cannot be entirely attributed to increased synthesis; they may well be due to a blocking of citrate metabolism in some manner.

Cohn and Forscher (12) and Cohn (10, 11) have studied the production of labeled carbon dioxide from Krebs and Glycolytic Cycle intermediates and the incorporation of the isotope into citrate by rabbit femur slices. They have reported that treatment with hormone did not affect the labeling of citrate derived from labeled pyruvate or glucose. The production of labeled carbon dioxide from radioactive citrate was reduced more than 60% than that from comparable controls. Production of labeled carbon dioxide from other Krebs Cycle intermediates such as succinate and fumarate were also reduced. However, the release of labeled carbon dioxide from glucose, pyruvate and lactate was increased by almost 40% in bone slices obtained from parathyroid extract-treated animals.

Mecca et al. found a decreased release of labeled carbon dioxide from radioactive citrate in parathyroid extract-treated calvaria cultures. Studies with radioactive glucose and pyruvate showed no effect in the release of labeled carbon dioxide when parathyroid extract-treated cultures were compared to the controls. They also prepared homogenates from calvaria

cultures and used them to study the release of labeled carbon dioxide from citrate. No differences were detected between control and parathyroid extract-treated calvaria homogenates until nicotinamide adenine dinucleotide phosphate was added to the bone preparation. At this addition a decrease in the release of labeled carbon dioxide from parathyroid extract-treated calvaria was noted. Nicotinamide adenine dinucleotide could not substitute for nicotinamide adenine dinucleotide phosphate, nor did it cause a further increase in the release of labeled carbon dioxide in homogenates already fortified with nicotinamide adenine dinucleotide phosphate. The addition of nicotinamide adenine dinucleotide phosphate was probably required because the preparation of the homogenates no doubt disrupted the cellular organization, not because of any altered parathyroid extract induced effect produced in the homogenate (41, 42).

Van Reen (58) investigated isocitric dehydrogenase activity in epiphyseal and metaphyseal preparations from rabbit bones and observed no significant differences in enzyme levels between control animals and animals receiving as much as 300 U.S.P. units of extract per kilogram of body weight. Parathyroid extract had been injected 16 - 18 hours prior to sacrifice. Aconitase activity was also measured in the same series of experiments, but no differences were detected. Van Reen also measured levels of nicotinamide adenine dinucleotide and nicotinamide

adenine dinucleotide phosphate in bone and found that parathyroid extract-treatment increased the concentration of these compounds markedly.

If parathyroid extract does not affect enzyme levels in bone, it might have its effect on a cofactor which is necessary for enzyme activity. Isocitric dehydrogenase activity in bone cannot be detected unless adequate nicotinamide adenine dinucleotide phosphate is added to the assay system which contains the enzyme extracted from the bone preparation. This suggests that the level of the coenzyme is the limiting factor in the system. The levels of NADase and NADPase found in the femurs of rabbits and dogs could be a factor in determining the amount of dinucleotide which is available to the enzyme. (60).

Hekkelman (28) was in apparent disagreement with Van Reen when he reported reduced isocitric dehydrogenase activity in rabbit femur preparations 16 hours after the injection of 100 U.S.P. units of parathyroid extract per kilogram of body weight. However, he agreed that there was no effect on aconitase activity. Later Hekkelman (29) reported the parathyroid extract effect on isocitric dehydrogenase activity is not on decreasing the concentration of the enzyme itself, but rather on the decreased extractability of the enzyme from bone. To explain the parathyroid extract effect on extractability of isocitric dehydrogenase, Hekkelman has postulated at least part of the isocitric dehydrogenase activity present in bone is bound to a cellular

particle. The enzyme is liberated only when the enzyme-nicotinamide adenine dinucleotide phosphate complex is formed. He believes that the presence of parathyroid hormone will reduce the amount of activated isocitric dehydrogenase complex formed by somehow limiting the amount of nicotinamide adenine dinucleotide phosphate available.

W.A. de Voogd van der Straaten (16) added parathyroid extract and nicotinamide adenine dinucleotide phosphate or compounds capable of increasing the intracellular nicotinamide adenine dinucleotide phosphate levels to bone tissue culture and found that the parathyroid extract-treated cultures fortified with nicotinamide adenine dinucleotide phosphate or a precursor demonstrated a reduced osteoclastic resorption. However,resorption was not completely eliminated in the fortified cultures. Evidence for a postulated parathyroid extract-induced increase of NADPase was also presented. He interpreted his data to mean that parathyroid extract could possibly cause a decreased biosynthesis of nicotinamide adenine dinucleotide phosphate or an increased enzymic breakdown via increased levels of NADPase.

In summary, it is believed the key to the mechanism of action of parathyroid hormone controlled plasma calcium homeostasis lies in the metabolic pathways of biological oxidation as observed in skeletal tissue and their relationship to bone resorption. The metabolic pathways under consideration include those of citric, lactic, succinic, and other organic acids.

Many believe that these acids may be important in the solubilization of bone mineral, and hence in the resorptive process. The production of citrate is of particular interest in connection with demineralization of bone because citrate ions chelate calcium, thus aiding in the solubilization of mineral (22, 26). Increased citrate levels observed after the administration of parathyroid hormone can be the result of increased production and/or an inhibition of oxidation. Current evidence favors the latter view. A change in the citrate level has to be the result of some change in the oxidative pathways. Since biological oxidations depend on the availability of suitable hydrogen acceptors, it has been of interest to study the coenzymes which usually serve this purpose, namely nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. The amount of available coenzyme could be a factor by which the oxidation processes are regulated. It appears that parathyroid hormone somehow reduces the amount of dinucleotide available for oxidative processes. This in turn limits the amount of activated nicotinamide adenine dinucleotide phosphate dependent enzyme, such as isocitric dehydrogenase, which is available for metabolism. Thus the proposed metabolic block is enacted and the acid products of metabolism accumulate and facilitate in the resorption of bone.

STATEMENT OF THE PROBLEM

Parathyroid hormone effects on intermediary metabolism in bone and kidney are currently under investigation in many laboratories. The results reported, particularly in the studies on bone, seem to vary both with the type of bone and tissue preparation used for the investigation. It is believed that the parathyroid-induced alterations in bone metabolism are associated with the overall mechanism of action of parathyroid hormone in the control of blood calcium homeostasis.

Yates et al. (62) demonstrated in vitro that under the influence of increased endogenous parathyroid hormone, femurs showed a general increase in the accumulation of citrate and lactate. The metaphysis was the site of greater lactate production; and the diaphysis was the site of greater citrate production.

This led us to believe that perhaps parathyroid hormone affects bone metabolism to a different extent or in a different manner in spongy and compact bone tissue. The study of the effect of parathyroid extract in epiphysis (trabecular bone) and diaphysis (compact bone) from the same femur might resolve some of the conflicts of experimental results obtained from various bone types reported in the literature.

In the approach employed in the research, two of the anatomically distinct regions of the femur, diaphysis and epiphysis, were employed in the experiments. Fragments and

crude homogenates of the two regions of the femur exised from parathyroid extract-treated and control rats were incubated with radioactively-labeled Krebs and Glycolytic Cycle intermediates. The preparations of epiphyses and diaphyses were used to determine whether the metabolism of these substrates by the tissues is influenced by the induced variation in parathyroid status of the animals.

CHAPTER II

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

Male Holtzman rats, approximately 150 - 170 grams in weight, were thyroparathyroidectomized. They were then allowed to eat ad libitum for sixty hours. The animals used in the experiments received two injections; experimental animals received 75 U.S.P. units of PTE (Lilly) and control animals received a comparable volume of vehicle at 24 and 48 hours after surgery. The vehicle injected was that used in the preparation of the extract and was composed of 1.6% glycerol and 0.2% phenol and 0.9% sodium chloride.

Approximately twelve hours following the second injection, blood samples were taken from the tails of control and experimental animals and were analyzed for calcium. In all cases, an average increase of 2 - 4 mg% was observed in the serum calcium of the experimental animals as compared to the controls.

A weighed portion of bone fragments prepared from control and experimental animals was added to four ml of incubation media which had been pipetted into a 25 ml incubation flask fetted with a disposable centerwell. Or, depending on the procedure involved, 4.0 ml of bone homogenate was pipetted into a 25 ml incubation flask. Fragments and homogenates were prepared from both the diaphyses and epiphyses of femurs obtained from control and experimental animals. A stream of 100% oxygen was

passed through each flask and radioactively labeled substrate together with cofactors was added to the flasks when needed.

The plastic centerwell containing 0.2 ml of 10% potassium hydroxide was set in place and the flask was stoppered. The incubation was carried out in a Dubnoff-type shaker water bath at a temperature of 37° C for a period of time ranging from one and one-half to three hours.

The incubation was stopped by the addition of 2 ml of 10% trichloroacetic acid to each flask. The 10% solution of potassium hydroxide contained in the centerwell was transferred to a culture tube by three washings with doubly distilled water. The labeled carbon dioxide absorbed in the potassium hydroxide solution was precipitated as barium carbonate and the radioactivity determined.

CHEMICALS

Barium chloride, Baker and Adamson, #1408, lot 6103

Calcein indicator, Fisher Scientific Company, #C-566, lot 79358

Calcium carbonate, Mallinckrodt Chemical Works, #4071

Calcium chloride, Mallinckrodt Chemical Works, lot 4160

DL Citric acid-1,5-^{14}C, Nuclear Chicago Corporation #CFA 263
batch 3, S.A. = 19.2mc/mM

Ethylenediaminetetraacetic acid

Fumaric Acid 1,4-^{14}C, Nuclear Chicago Corporation #CFA 67,
batch 9, S.A. = 23.0mc/mM

Filter paper

Whatman #50

Whatman # 1

D-Glucose-1-^{14}C, Nuclear Chicago Corporation, #CFA 72, batch 99, S.A. = 2.96 mc/mM

D-Glucose-2-^{14}C, Nuclear Chicago Corporation #CFA 73, batch 86 S.A. = 2.8 mc/mM

D-Glucose-6-^{14}C, Nuclear Chicago Corporation #CFA 205, batch 18, S.A. = 27 mc/mM

Glycerol, Mallinckrodt Chemical Works, #3076, lot 4221

Magnesium sulfate, Mallinckrodt Chemical Works, lot 6070

Nicotinamide adenine dinucleotide phosphate, Sigma Chemical Company, lot 95B-7920

Parathyroid Extract, gift, Eli Lilly Company

Phenol (carbolic acid), Mallinckrodt Chemical Works, lot 4644

Phosphoric acid, J.T. Baker Chemical Company, lot 0260

Potassium chloride, Mallinckrodt Chemical Works, lot 6858

Potassium hydroxide, 45% solution, J.T. Baker Chemical Company, lot 25027

Potassium iodide, Mallinckrodt Chemical Works, lot 2366

Sodium chloride, Mallinckrodt Chemical Works, lot 7581

Sodium cyanide, J.T. Baker Chemical Company, #1144

Sodium hydroxide, Mallinckrodt Chemical Works, lot 7708

Sodium 2-oxoglutarate, Nuclear Chicago Corporation, #CFA 139, Batch 16, S.A. = 9.33 mc/mM

Sodium DL-lactate-1-^{14}C, Nuclear Chicago Corporation, #CFA 90, batch 13, S.A. = 5.5 mc/mM

Sodium pyruvate-2-^{14}C, Nuclear Chicago Corporation, #CFA 79, batch 55, S.A. = 10.0 mc/mM

Succinic acid-2,3-^{14}C, California Corporation for Biochemical Research, lot 880093, S.A. = 9.6 mc/mM

Trichloracetic acid, J.T. Baker Chemical Company, #0414

SERUM CALCIUM DETERMINATION

A method of determining ionizable calcium in serum, which is essentially unaffected by organic materials present, has been reported by Ashby and Roberts (4). This method is based upon the fact that at pH's above 12, calcein (an iminoacetate derivative of fluorescein) fluoresces under long-wave ultraviolet light only in the presence of free calcium. In an analysis, a given amount of ethylenediaminetetraacetic acid (EDTA) which is in excess of that required to complex all of the calcium present is added to the sample. A small amount of cyanide is added to complex any copper or iron which is present. The solution is back-titrated with standard calcium solution with the calcium replacing the magnesium in any magnesium-EDTA complex which might have formed. When all of the EDTA has become complexed with calcium, additional calcium will combine with calcein causing fluorescence, and the end point will have been reached. Titrations must also be performed on calcium-free samples in order to determine the total calcium binding

capacity of the EDTA and the amount of calcium required to titrate the serum sample.

Four hundred microliters of blood sample were obtained from each rat by tail bleeding. The samples were placed in the refrigerator for a short time before the clot was separated by a five minute centrifugation in a Beckman/Spinco microfuge. Thirty microliter aliquots of serum were added to microtitrator cups. To each cup was added 0.150 ml of 0.002 M EDTA, one drop (about 60 ul) of dilute calcein indicator, 1 drop (about 30 ul) of 1% sodium cyanide solution, and 1 drop (about 60 ul) of 1.0 N sodium hydroxide. Titration was performed with a microtitrator containing a 20.0 mg% standard calcium solution. A long-wave ultraviolet lamp (Mineralight, Model SL 3660) was placed about 2" above the titration cup. The standard calcium solution was added with continuous stirring until the green fluorescence which was observed no longer increased in intensity. The calcium concentration of the sample is directly proportional to the difference in microliters of standard calcium required to titrate the sample and a water blank. A 10.0 mg% standard calcium solution was run with each group of samples and the calcium concentrations were calculated as follows:

$$\text{mgs\% calcium} = 10 \text{ mg\%} \times \frac{\text{microliters for unknown}}{\text{microliters for 10 mg\% standard}}$$

Where:

microliters unknown = microliters of titrant required to titrate blank minus microliters of titrant required to titrate unknown sample

microliters 10 mg% standard = microliters of titrant required to titrate blank minus microliters of titrant required to titrate 10 mg% standard

Figure I indicates the linear relationship between the microliters of titrant used and the concentration of calcium in solution. This is the standard calcium titration curve used to calculate serum calcium concentration for the animals used in the experiments. The data for this standard curve appears in Table I.

TABLE I

STANDARD TITRATION CURVE DATA FOR CALCIUM DETERMINATION

Actual Calcium	Number of Samples	Microliters of Titrant	Microliters (Titrant - 0 mg%)
0 mg%	6	30.7 ± 0.2	
6	6	20.6 ± 0.3	10.2
8	6	17.3 ± 0.4	13.4
10	6	13.9 ± 0.3	16.8
12	6	10.8 ± 0.5	19.9
14	6	7.5 ± 0.2	23.3
16	6	4.9 ± 0.3	25.9

Solutions:

Calcein solution-concentrated. 0.25 g of powdered indicator was dissolved in 4.0 ml of 1.0 N NaOH. When solution was complete this was diluted to 100 ml with doubly distilled water.

Calcein indicator solution. 0.5 ml of the concentrated calcein solution was diluted to 25 ml with doubly distilled water.

Calcium standard solution. 0.2497 g of oven-dried calcium carbonate was dissolved in 30 ml of 2 N HCl and diluted to 500 ml with doubly distilled water. Working solutions were prepared from this stock solution.

Ethylenediaminetetraacetic acid standard. 0.375 g of EDTA dissolved in CO_2-free water to a total volume of 1000 ml.

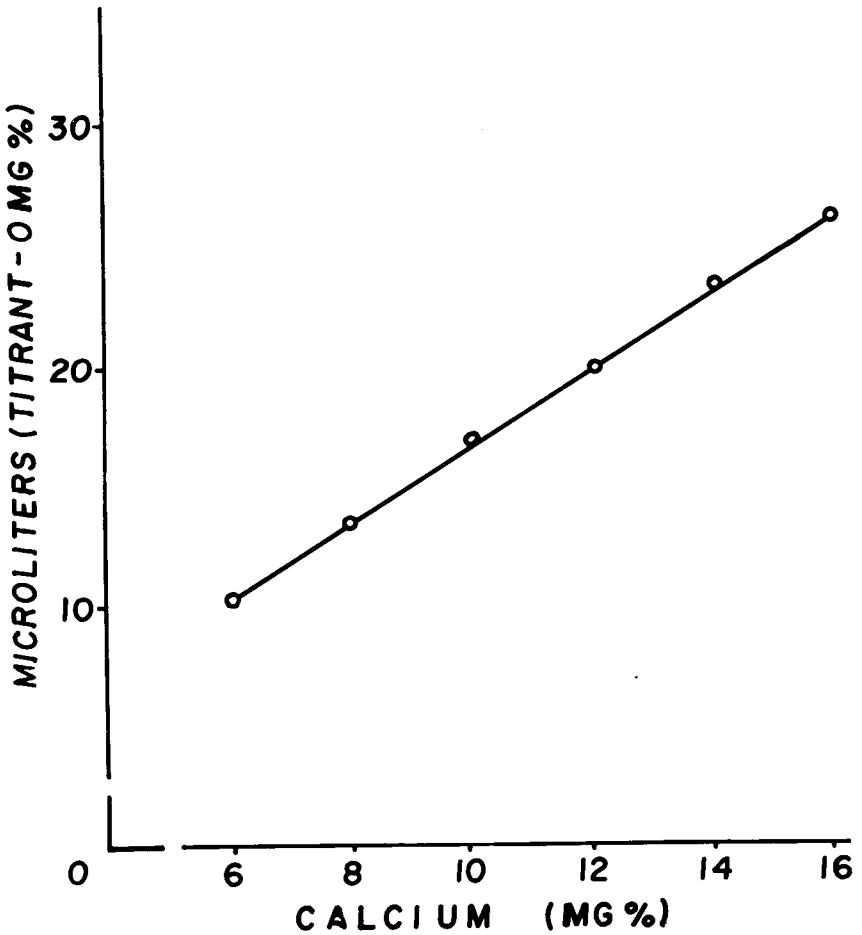

FIGURE 1

STANDARD CURVE FOR CALCIUM DETERMINATION

BLOOD SAMPLING

Blood samples were obtained from the tail of the rat. The rat was placed in a restraining cage and the tail severed approximately three quarters of an inch from the tip. Bleeding was aided by "milking" the tail in a proximal to distal direction. Blood was collected in plastic microtubes, and these were allowed to stand at room temperature for thirty minutes to one hour to allow for clot formation and retraction. Centrifugation was performed in a Beckman/Spinco Microfuge for two minutes. Supernatant serum was separated from centrifugate and stored under refrigeration in a microtube until analyzed.

THYROPARATHYROIDECTOMY

Male Holtzman albino rats were anesthetized with Nembutal. The dose administered was 30 mg of Nembutal per mg. of body weight, and it maintained the rats at a proper level of anesthesia for an hour or longer. The anesthetic takes effect within five to ten minutes after administration.

After the rat ceased struggling it was secured on an operating board. The neck of the rat was held taut by a rubber band placed around the upper incisors of the rat and two suitably located nails in the operating board.

An incision about one inch long was made along the midline of the neck. The skin was pulled free from the underlying musculature and the tissue teased apart parallel to the trachea with forceps in order to expose the musculature overlying

the trachea. In this procedure, care must be taken not to cut into or bruise the salivary glands, as this will cause profuse bleeding. Forceps were used to tease apart the striated muscle which surrounds the trachea. The musculature of the trachea was retracted with forceps and the trachea exposed.

In the rat, the parathyroid glands are sometimes visible as lighter colored areas at the upper ends of the thyroids. However, they are usually impossible to discern with the naked eye. The entire thyroid-parathyroid apparatus was teased away from the trachea with forceps. Usually the two thyroids with connecting isthmus can be removed intact if care is exercised in the operation. It is desirable to remove the organ intact when possible, for this insures completeness of removal and induces less bleeding.

Once the glands are removed, a cotten swab is placed in the area until bleeding ceases. The cotten swab and hemostats are then removed and the wound is closed with two or three stainless steel wound clips. The time for the operation averaged ten minutes.

The animals are returned to the cages for recovery. One day later a check is made to determine if any of the animals have developed ataxic breathing. Those that had were destroyed.

THE PREPARATION OF FRAGMENTS AND HOMOGENATES FROM RAT FEMUR

Male Holtzman rats were stunned by a sharp blow to the head and decapitated. A semicircular incision was made around

the leg just below the pelvis. The skin was pulled toward the foot exposing the musculature of the thigh area. The tendon connecting the epiphysis and a portion of the musculature was severed. The severed tendon was grasped with forceps and pulled toward the pelvis. This procedure tears away most of the ventral musculature of the thigh and exposes the femur. The remaining musculature was scraped away with a scalpel. The femur was severed from the tibia and worked free from the socket in the pelvis.

The extirpated femur was placed in ice-cold incubation media. The bone was cleaned of all adherent muscle, periosteum, and cartilage. The distal epiphysis is separated from the diaphysis. The proximal epiphysis is also separated from the diaphysis, but it is discarded. The distal epiphysis and diaphysis were split longitudinally and the marrow removed by scraping with the point of a scalpel and washing with isotonic saline.

For the preparation of bone fragments, the sections of epiphysis and diaphysis were blotted dry and weighed on a Roller-Smith balance. Approximately 150 - 200 mg of the split bone sections were fragmentized on glass or lucite plate using a stainless steel scalpel and placed in a twenty-five ml incubation flask containing 4.0 ml of incubation media.

For the preparation of homogenates, sections of epiphysis and diaphysis were placed in a bone disintegrinder (Kontes)

with 10.0 - 30.0 ml of incubation media depending on the amount of bone to be homogenized. Homogenization was performed for five minutes or until no further contact between the disintegrinder and bone can be felt.

Homogenates have a milky appearance and settle out rapidly. While pipetting, the homogenate is kept agitated with a magnetic stirrer. Whenever possible tissue was maintained at a temperature of 0° C during preparation.

An estimation of the amount of material in any particular volume of homogenate was obtained by pipetting a 1.0 ml aliquot of homogenate on a preweighed planchet. The aliquot was dried on the planchet using a heat lamp. The planchet was weighed and the dry weight of the material in the aliquot determined by the weight difference of the planchet. This was necessary so that the data could be analyzed according to $C^{14}O_2$ evolved from substrate metabolized by an estimated amount (dry weight) of bone present in the homogenate.

PREPARATION OF MEDIA

The medium used in these experiments was prepared by mixing 232 ml 0.154 M NaCl, 8 ml 0.154 M KCl, 6 ml 0.110 M $CaCl_2$, and 2 ml 0.154 M $MgSO_4$, and adding 25 ml of a phosphate buffer prepared by bringing 75 ml of 0.2 N NaOH to a pH of 7.4 with 2 M H_3PO_4 and diluting to 100 ml with doubly distilled water. All solutions were prepared with doubly distilled water.

LABELED CARBON DIOXIDE COLLECTION AND MEASUREMENT

Radioactive carbon dioxide produced by the oxidation of the radioactively labeled substrate in the bone tissue preparation was absorbed in 0.2 ml of a 10% solution of potassium hydroxide contained in the centerwell of the incubation flask. The centerwell of the flask also contained a small fold of Whatman #1 filter paper. The purpose of the paper was to increase the surface area of contact of the potassium hydroxide solution and to help control movement of the liquid during the incubation in the shaker bath.

Once the incubation is ended, the filter paper in the centerwell was transferred to a test tube. Five ml of CO_2 free distilled water were added to each tube and the tubes were transferred to another test tube which contained 5 ml of 10% barium chloride solution. The tubes were allowed to stand at room temperature until the white, flocky, precipitate settled to the bottom of the tube.

The precipitate of barium carbonate was transferred to a Tracerlab Precipitation Apparatus, Model E-8B which was fitted with a disk of Whatman #50 filter paper. Suction was applied and the radioactive barium carbonate was plated on the disk of filter paper. The plated precipitate was washed two times with water, 50 - 50 (v/v) ethanol-acetone, and acetone. The plated samples were allowed to dry in air for two hours before weighing and counting.

The counting was accomplished with a thin end-window type assembly consisting of a Tracerlab Tracermatic SC-83 Scaler, Tracerlab SC-100 Multi-matic Sample Changer, and a Tracerlab SC-66 Printing Timer. Corrections for self-absorption and dead time were made. The corrections for self-absorption were made according to the following formula (61):

$$\frac{I}{I_o} = \frac{1 - e^{-uh}}{uh}$$

Where:

I = observed activity

I_o = actual activity

u = 0.29

h = mg/cm^2 of plated material

The self-absorption correction curve appears in Figure 2. The correction curve is based upon data appearing in Table II.

TABLE II

$BaC^{14}O_3$ SELF ABSORPTION DETERMINATION

$BaC^{14}O_3$ mg	Correction	$BaC^{14}O_3$ mg	Correction
0.0-0.5	1.00	14.0-14.5	0.52
0.5-1.0	0.95	14.5-15.0	0.51
1.0-1.5	0.93	15.0-15.5	0.50
1.5-2.0	0.91	15.5-16.0	0.49
2.0-2.5	0.89	16.0-16.5	0.48
2.5-3.0	0.88	16.5-17.0	0.47
3.0-3.5	0.86	17.0-17.5	0.46
3.5-4.0	0.82	17.5-18.0	0.45
4.0-4.5	0.80	18.0-18.5	0.44
4.5-5.0	0.78	18.5-19.0	0.43
5.0-5.5	0.76	19.0-19.5	0.43
5.5-6.0	0.74	19.5-20.0	0.42
6.0-6.5	0.73	20.0-20.5	0.42
6.5-7.0	0.71	20.5-21.0	0.41
7.0-7.5	0.70	21.0-21.5	0.41
7.5-8.0	0.68	21.5-22.0	0.40
8.0-8.5	0.67	22.0-22.5	0.39
8.5-9.0	0.66	22.5-23.0	0.39
9.0-9.5	0.65	23.0-23.5	0.38
9.5-10.0	0.64	23.5-24.0	0.38
10.0-10.5	0.62	24.0-24.5	0.37
10.5-11.0	0.61	24.5-25.0	0.37
11.0-11.5	0.60	30.0	0.32
11.5-12.0	0.58	35.0	0.29
12.0-12.5	0.57	40.0	0.26
12.5-13.0	0.56	45.0	0.23
13.0-13.5	0.55	50.0	0.21
13.5-14.0	0.53		

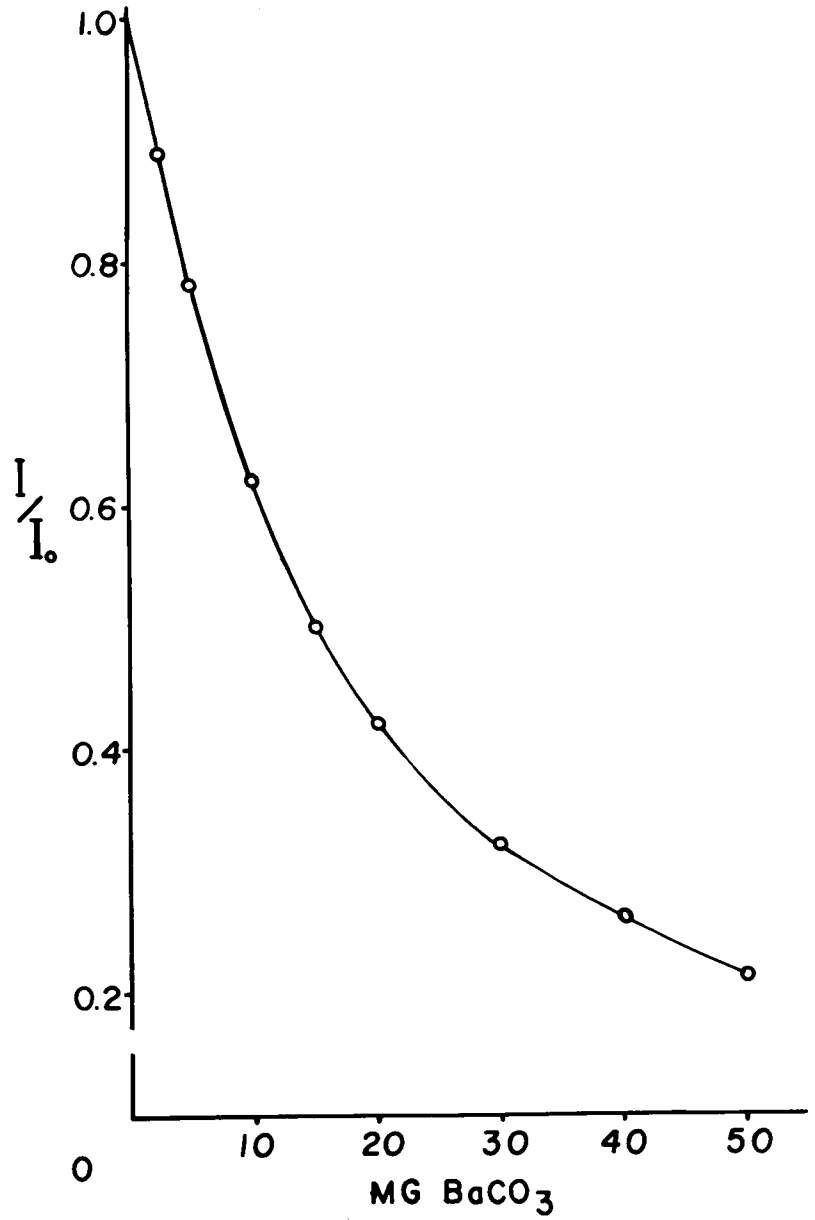

FIGURE 2

SELF-ABSORPTION CORRECTION CURVE FOR $Ba^{14}CO_3$

CHAPTER III

EXPERIMENTAL RESULTS

The experiments utilized the detection of $^{14}CO_2$ evolution from radioactively labeled substrate as a measure of *in vitro* oxidation of substrate by bone tissue preparations. The substrates used in the experiments include citrate-1,5-^{14}C, succinate-2,3-^{14}C, fumarate-1,4-^{14}C, 2-oxoglutarate-5-^{14}C, pyruvate-2-^{14}C, lactate-1-^{14}C, glucose-6-^{14}C, glucose-2-^{14}C, and glucose-1-^{14}C. The oxidation of these substrates was studied using fragments, homogenates, and NADP-fortified homogenates, prepared from femurs excised from parathyroid extract-treated and control animals, all of which were previously thyroparathyroidectomized.

Each of the experiments indicates the oxidation detected in the diaphyses and the epiphyses of the femurs utilized. Data is presented for each flask used in the incubation studies. The average change in the oxidation by the diaphyses and epiphyses of the PTE-treated and control animals is calculated for each experiment. The parathyroid status of the experimental and control animals was assessed on the basis of their serum calcium levels.

Experiments 1 and 2: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,5-^{14}C

Six thyroparathyroidectomized rats were used in each

experiment. Three of the rats were injected with parathyroid extract and three injected with vehicle. The rats which received 150 U.S.P. units of extract showed an average increase in serum calcium of 3.1 mg% in experiment 1 and an increase of 2.9 mg% in experiment 2.

In experiment 1, 0.5 microcurie of radioactive substrate 150 - 200 mg of bone fragments and 4.0 ml of incubation media were present in each flask. The incubation was carried out for 2 hours at 37° C.

The results appear in Table III. The diaphysis of the femur showed an average depression of 65% in the oxidation of substrate by the bone preparation obtained from the PTE-treated rats when compared with controls. The epiphysis of the femur demonstrated an average 65% increase in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated rats when compared to controls.

In experiment 2 the amount of substrate was increased from one-half to one microcurie per flask, but the amount of bone fragments was not changed. It was noted that the total amount of oxidation as measured by counts per minute of radioactivity remained about the same in both experiments. This could be caused by a limited amount of metabolizing tissue present in the bone fragments.

In reference to the latter factor bone is composed of nearly two thirds mineral crystal by weight. The organic matrix

in which the mineral crystal is deposited is composed of the protein collagen, and it too is in a crystalline state. Therefore the small number of bone cells per mg of bone tissue preparation could limit the amount of oxidation detected regardless of the amount of radioactive substrate available to the system.

As noted in Table IV, an average decrease of 50% in the oxidation of citrate-1,5^{14}C by the bone preparation was obtained from the PTE-treated animals compared to the controls. The epiphysis demonstrated a 75% average increase in the oxidation of the substrate by the bone preparation obtained from the PTE-treated animals.

Experiment 3 and 4: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Succinate-2,3-^{14}C

Eight thyroparathyroidectomized rats were used in experiment 3. Four of the rats were injected subcutaneously with parathyroid extract, and four were injected subcutaneously with vehicle. The rats which received 150 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 2.7 mg% over that of the control animals.

Two microcuries of succinate-2,3^{14}C, 200 mg of bone fragments and 4.0 ml of incubation media were present in each of the incubation flasks. The incubation was run for three and one half hours at 37° C in a Dubnoff-type shaker water bath.

TABLE III

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	4,200	- 65%
Citrate-1,5-^{14}C	PTE-Treatment	4,800	
Citrate-1,5-^{14}C	Control	7,200	
Citrate-1,5-^{14}C	Control	7,100	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	7,000	+ 65%
Citrate-1,5-^{14}C	PTE-Treatment	6,600	
Citrate-1,5-^{14}C	Control	4,300	
Citrate-1,5-^{14}C	Control	5,100	

TABLE IV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	3,300	- 50%
Citrate-1,5-^{14}C	PTE-Treatment	4,700	
Citrate-1,5-^{14}C	Control	7,800	
Citrate-1,5-^{14}C	Control	7,400	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	3,900	+ 75%
Citrate-1,5-^{14}C	PTE-Treatment	4,500	
Citrate-1,5-^{14}C	Control	2,700	
Citrate-1,5-^{14}C	Control	2,100	

The diaphysis of the femur showed an average depression of 30% in the oxidation of the substrate by the bone preparation obtained from the PTE-treated animals when compared to the control animals. The epiphysis of the femur produced an average increase of 45% in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated animals when compared to the control animals. (Table V).

Experiment 4 was prepared and carried out exactly as was experiment 3. The rats which received 150 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 2.4 mg% over that of the control animals.

The diaphysis of the femur demonstrated an average depression of 25% in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated animals when compared to the control animals. The epiphysis of the femur showed a 33% average increase in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated rats when compared to the control animals. (Table VI).

Experiment 5: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Fumarate-1,4-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium

TABLE V

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	5,900	
Succinate-2,3-^{14}C	PTE-Treatment	6,000	- 30%
Succinate-2,3-^{14}C	PTE-Treatment	6,300	
Succinate-2,3-^{14}C	Control	9,500	
Succinate-2,3-^{14}C	Control	8,800	
Succinate-2,3-^{14}C	Control	7,600	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	17,500	
Succinate-2,3-^{14}C	PTE-Treatment	14,200	+ 45%
Succinate-2,3-^{14}C	PTE-Treatment	14,400	
Succinate-2,3-^{14}C	Control	10,500	
Succinate-2,3-^{14}C	Control	11,100	
Succinate-2,3-^{14}C	Control	11,000	

TABLE VI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	3,800	
Succinate-2,3-^{14}C	PTE-Treatment	3,800	
Succinate-2,3-^{14}C	PTE-Treatment	3,400	- 25%
Succinate-2,3-^{14}C	PTE-Treatment	3,000	
Succinate-2,3-^{14}C	Control	8,600	
Succinate-2,3-^{14}C	Control	10,000	
Succinate-2,3-^{14}C	Control	9,000	
Succinate-2,3-^{14}C	Control	9,800	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	8,600	
Succinate-2,3-^{14}C	PTE-Treatment	10,000	
Succinate-2,3-^{14}C	PTE-Treatment	9,000	+ 33%
Succinate-2,3-^{14}C	PTE-Treatment	9,800	
Succinate-2,3-^{14}C	Control	6,300	
Succinate-2,3-^{14}C	Control	4,900	
Succinate-2,3-^{14}C	Control	7,000	

of 2.6 mg%.

One microcurie of radioactive substrate, 4.0 ml of incubation media and 150 mg of tissue were present in each flask. The incubation was carried out for three hours at 37° C.

The diaphyses of the femora showed an average decrease of 25% in the oxidation of the substrate by bone preparation obtained from the PTE-treated animals. The epiphyses of the femora showed an average increase of 85% in the oxidation of the substrate by bone preparations obtained from the PTE-treated animals. (Table VII).

Experiment 6: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with 2-Oxoglutarate-1-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.9 mg% above the control animals.

One microcurie of radioactive substrate, 4.0 ml of incubation media and 150 mg of tissue were present in each flask. The incubation was carried out for three hours at 37° C.

The diaphyses of the femora demonstrated an average decrease of 40% in the oxidation of the substrate by the bone preparation obtained from the PTE-treated animals. The epiphyses of the femora showed a slight increase in the oxidation of

TABLE VII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF FUMARATE-1,4-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Fumarate-1,4-^{14}C	PTE-Treatment	13,400	- 25%
Fumarate-1,4-^{14}C	PTE-Treatment	9,800	
Fumarate-1,4-^{14}C	Control	21,000	
Fumarate-1,4-^{14}C	Control	18,400	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Fumarate-1,4-^{14}C	PTE-Treatment	25,400	+ 85%
Fumarate-1,4-^{14}C	PTE-Treatment	26,800	
Fumarate-1,4-^{14}C	Control	14,000	
Fumarate-1,4-^{14}C	Control	13,600	

TABLE VIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF 2-OXOGLUTARATE-5-^{14}C BY BONE FRAGMENTS OF DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
2-Oxoglutarate-5-^{14}C	PTE-Treatment	2,800	- 40%
2-Oxoglutarate-5-^{14}C	PTE-Treatment	3,400	
2-Oxoglutarate-5-^{14}C	Control	4,900	
2-Oxoglutarate-5-^{14}C	Control	5,800	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
2-Oxoglutarate-5-^{14}C	PTE-Treatment	23,000	+ 12%
2-Oxoglutarate-5-^{14}C	PTE-Treatment	29,000	
2-Oxoglutarate-5-^{14}C	Control	22,700	
2-Oxoglutarate-5-^{14}C	Control	26,800	

the substrate by bone preparations obtained from the PTE-treated animals. (Table VIII).

Experiment 7: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Pyruvate-2^{14}C

Six thyroparathyroidectomized rats were used in the experiment. Three of the rats were injected subcutaneously with 150 U.S.P. units of parathyroid extract, and three were injected subcutaneously with vehicle. The rats which were injected with parathyroid extract showed an average increase in serum calcium of 2.8 mg% over that of the control animals injected with vehicle.

Two microcuries of radioactive substrate, 100 mg of bone fragments, and 4.0 ml of media were present in each incubation flask. The incubation was performed for three hours at 37° C.

It can be seen from the data presented in Table IX that the diaphysis of the femora showed an average increase of 75% in the oxidation of substrate by the bone preparation obtained from the PTE-treated animals when compared to the control animals. The epiphysis demonstrated a 40% average increase in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated rats when compared to the control animals.

TABLE IX

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	49,000	
Pyruvate-2-^{14}C	PTE-Treatment	38,200	+ 75%
Pyruvate-2-^{14}C	PTE-Treatment	37,000	
Pyruvate-2-^{14}C	PTE-Treatment	36,700	
Pyruvate-2-^{14}C	Control	26,400	
Pyruvate-2-^{14}C	Control	22,200	
Pyruvate-2-^{14}C	Control	29,000	
Pyruvate-2-^{14}C	Control	20,300	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	100,000	
Pyruvate-2-^{14}C	PTE-Treatment	78,000	+ 42%
Pyruvate-2-^{14}C	PTE-Treatment	80,000	
Pyruvate-2-^{14}C	PTE-Treatment	92,000	
Pyruvate-2-^{14}C	Control	71,000	
Pyruvate-2-^{14}C	Control	56,400	
Pyruvate-2-^{14}C	Control	57,400	
Pyruvate-2-^{14}C	Control	61,000	

Experiment 8: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Lactate-1-^{14}C

Six thyroparathyroidectomized rats were used in the experiment. Three of the rats were injected subcutaneously with parathyroid extract, and three were injected subcutaneously with vehicle. The rats which received 150 units of parathyroid extract showed an average increase in serum calcium of 3 mg%.

Two microcuries of radioactive substrate with 100 mg of diaphysis plus 200 mg of epiphysis were present in their respective incubation flasks. The incubation was carried out for three hours at a temperature of 37° C.

Neither the diaphysis or the epiphysis showed any significant differences when the oxidation of the substrate by the bone preparations obtained from PTE-treated animals was compared with those obtained from the control animals. (Table X).

Bone cells are distributed unevenly throughout the bone tissue. Ossification centers and growing areas of bone will have a denser cell population than older, completely mineralized areas of bone. In the preparation of bone fragments the possibility exists of inadvertently "loading" an incubation flask with tissue containing a preponderance of living cells and another with tissue containing a preponderance of non-living mineral. Also, the radioisotope must penetrate the intricate canals in the fragmentized haversian system of the rat femur in

TABLE X

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	17,100	
Lactate-1-^{14}C	PTE-Treatment	14,000	No Change
Lactate-1-^{14}C	PTE-Treatment	14,400	
Lactate-1-^{14}C	PTE-Treatment	17,700	
Lactate-1-^{14}C	Control	16,900	
Lactate-1-^{14}C	Control	14,300	
Lactate-1-^{14}C	Control	14,300	
Lactate-1-^{14}C	Control	22,300	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	29,400	
Lactate-1-^{14}C	PTE-Treatment	48,400	No Change
Lactate-1-^{14}C	PTE-Treatment	62,000	
Lactate-1-^{14}C	PTE-Treatment	48,700	
Lactate-1-^{14}C	Control	52,100	
Lactate-1-^{14}C	Control	50,700	
Lactate-1-^{14}C	Control	49,100	

order to come into contact with a living cell.

Attempts were made to prepare homogenates of femur through the use of a Kontes bone disintegrinder. The homogenate prepared was coarse and had to be kept agitated or the milky suspension would rapidly settle out. As a measure of homogenate homogeneity, one ml aliquots were pipetted onto preweighed planchets and dried. The dried aliquots had a weight variation of ± 0.20 mg in 20 mg total weight of bone homogenate.

Experiments 9 and 10: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,5-^{14}C

Two thyroparathyroidectomized rats were used in both experiments 9 and 10. One rat was injected subcutaneously with parathyroid extract, and the control rat was injected subcutaneously with vehicle. The rats which received 162 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 2.1 mg% in experiment 9 and 2.4 mg% in experiment 10.

In experiment 9, 0.5 microcurie of radioactive substrate and 4.0 ml of homogenate were present in each incubation flask. The incubation was run for three hours at 37° C.

Femur diaphysis and epiphysis showed no significant differences in the oxidation of substrate by bone preparation obtained from the PTE-treated rats when compared to controls. (Table XI).

In experiment 10, 1.0 microcurie of citrate-1,5^{14}C

substrate and 4.0 ml of homogenate were present in each incubation flask. The incubation was performed for three hours at 37° C in a Dubnoff-type shaker water bath.

Femur diaphysis and epiphysis showed no significant differences in the oxidation of substrate by bone preparation obtained from the PTE-treated rats when compared with controls. (Table XII).

Unlike the incubation of citrate-1,5-^{14}C with bone fragments an increase from one half to one microcurie per flask produced an increase of almost 100% in the production of labeled carbon dioxide in each flask. This seems to indicate the physical barriers that appeared to have existed in the experiment using bone fragments with citrate-1,5-^{14}C seem to have been eliminated in the experiments utilizing homogenates.

Experiment 11: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Succinate-2,3-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with parathyroid extract, and two were injected subcutaneously with vehicle. The animals which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.9 mg%.

One-half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The experiment was

TABLE XI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	2,250	No Change
Citrate-1,5-^{14}C	PTE-Treatment	2,150	
Citrate-1,5-^{14}C	Control	2,050	
Citrate-1,5-^{14}C	Control	1,950	

EPIPHYSIS Substrate	Experimental Condition	Acitvity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	3,300	No Change
Citrate-1,5-^{14}C	PTE-Treatment	3,350	
Citrate-1,5-^{14}C	Control	3,250	
Citrate-1,5-^{14}C	Control	3,100	

TABLE XII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	1,300	No Change
Citrate-1,5-^{14}C	PTE-Treatment	1,100	
Citrate-1,5-^{14}C	Control	1,100	
Citrate-1,5-^{14}C	Control	1,200	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	1,800	No Change
Citrate-1,5-^{14}C	PTE-Treatment	1,700	
Citrate-1,5-^{14}C	Control	1,700	
Citrate-1,5-^{14}C	Control	1,650	

run for three hours at 37° C.

No significant differences were noted in either the diaphysis or the epiphysis as is evident from Table XIII.

Experiment 12 and 13: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Pyruvate-2-^{14}C

Four thyroparathyroidectomized rats were used in each experiment. Two of the rats were injected subcutaneously with parathyroid extract, and two were injected subcutaneously with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 1.8 mg% in experiment 12 and 2.5 mg% in experiment 13 over the respective control animals.

One-half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask in experiment 12. The incubation was carried out for three hours at 37° C.

The diaphyses of the femora showed an average increase of 40% in the oxidation of pyruvate-2-^{14}C by the bone preparation obtained from the PTE-treated animals when compared to the control animals. The epiphyses of the femora showed an average increase of 15% in the oxidation of the substrate by preparations obtained from the PTE-treated animals when compared to the controls. (Table XIV).

One microcurie of radioactive substrate and 4.0 ml of homogenate were present in each incubation flask in experiment

TABLE XIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	930	
Succinate-2,3-^{14}C	PTE-Treatment	870	No Change
Succinate-2,3-^{14}C	PTE-Treatment	860	
Succinate-2,3-^{14}C	PTE-Treatment	840	
Succinate-2,3-^{14}C	Control	950	
Succinate-2,3-^{14}C	Control	740	
Succinate-2,3-^{14}C	Control	860	
Succinate-2,3-^{14}C	Control	920	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	2,740	
Succinate-2,3-^{14}C	PTE-Treatment	2,990	No Change
Succinate-2,3-^{14}C	PTE-Treatment	2,890	
Succinate-2,3-^{14}C	PTE-Treatment	2,760	
Succinate-2,3-^{14}C	Control	2,730	
Succinate-2,3-^{14}C	Control	2,640	
Succinate-2,3-^{14}C	Control	2,790	
Succinate-2,3-^{14}C	Control	2,850	

13. The incubation was performed for three hours at 37° C in a Dubnoff-type shaker water bath.

The diaphyses of the femora showed an average increase of 50% in the oxidation of the radioactive substrate by bone preparations obtained from the PTE-treated animals. The epiphyses of the femora showed an average increase of 30% in the oxidation of pyruvate-2-^{14}C by bone homogenate obtained from the PTE-treated animals when compared to the control animals. (Table XV).

Experiments 14, 15, and 16: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Lactate-1-^{14}C

Four thyroparathyroidectomized rats were used in experiments 14 and 15. Two thyroparathyroidectomized rats were used in experiment 16. Two out of the four and one out of the two, respectively were injected subcutaneously with parathyroid extract. The rats which received a total of 162 U.S.P. units over a period of 48 hours showed an average increase in serum calcium of 2.7, 2.6 and 2.8 mg% respectively in experiments 14, 15 and 16.

One microcurie of radioactive substrate with 4.0 ml of homogenate was present in each incubation flask in experiment 14 and 15. The only modification in experiment 16 is that only 0.5 microcurie of radioactive substrate was present in each incubation flask. The incubation was performed for three hours at 37° C.

TABLE XIV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	39,200	
Pyruvate-2-^{14}C	PTE-Treatment	40,500	+ 40%
Pyruvate-2-^{14}C	PTE-Treatment	48,000	
Pyruvate-2-^{14}C	PTE-Treatment	42,100	
Pyruvate-2-^{14}C	Control	28,800	
Pyruvate-2-^{14}C	Control	29,200	
Pyruvate-2-^{14}C	Control	29,200	
Pyruvate-2-^{14}C	Control	27,300	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	69,000	
Pyruvate-2-^{14}C	PTE-Treatment	70,000	+ 15%
Pyruvate-2-^{14}C	PTE-Treatment	74,600	
Pyruvate-2-^{14}C	PTE-Treatment	69,500	
Pyruvate-2-^{14}C	Control	61,300	
Pyruvate-2-^{14}C	Control	60,300	
Pyruvate-2-^{14}C	Control	59,900	
Pyruvate-2-^{14}C	Control	61,300	

TABLE XV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	62,000	
Pyruvate-2-^{14}C	PTE-Treatment	54,100	+ 50%
Pyruvate-2-^{14}C	PTE-Treatment	58,000	
Pyruvate-2-^{14}C	Control	42,000	
Pyruvate-2-^{14}C	Control	35,500	
Pyruvate-2-^{14}C	Control	37,500	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	154,000	
Pyruvate-2-^{14}C	PTE-Treatment	141,000	+ 30%
Pyruvate-2-^{14}C	PTE-Treatment	143,500	
Pyruvate-2-^{14}C	Control	111,000	
Pyruvate-2-^{14}C	Control	111,500	
Pyruvate-2-^{14}C	Control	111,500	

Both diaphysis and epiphysis showed variable differences in all three experiments when the oxidation of the substrate by the bone preparation obtained from the PTE-treated animals was compared to oxidation by the bone preparation obtained from the control animals (Tables XVI,XVII, and XVIII).

Experiment 17: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Lactate-2-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the animals were injected subcutaneously with parathyroid extract and the other two animals were injected subcutaneously with the vehicle used in the preparation of the extract. The rats which received 162 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 2.2 mg% over that of the control animals.

Four mls of bone homogenate were present in each incubation flask which contained 0.5 microcurie of the radioactive substrate. The incubation was performed for three hours in a Dubnoff-type shaker water bath at a temperature of 37° C.

An average stimulation of 62% in the oxidation of the radioactive substrate by bone homogenate obtained from the PTE-treated animals when compared to the controls was demonstrated by the diaphyses. The epiphyses showed an average depression of 15% in the oxidation of the radioactive substrate by bone homogenates obtained from the PTE-treated animals when compared

TABLE XVI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	4,900	
Lactate-1-^{14}C	PTE-Treatment	4,300	No Change
Lactate-1-^{14}C	PTE-Treatment	4,700	
Lactate-1-^{14}C	Control	5,300	
Lactate-1-^{14}C	Control	5,000	
Lactate-1-^{14}C	Control	5,400	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	17,600	
Lactate-1-^{14}C	PTE-Treatment	16,900	No Change
Lactate-1-^{14}C	PTE-Treatment	15,600	
Lactate-1-^{14}C	Control	14,100	
Lactate-1-^{14}C	Control	17,250	
Lactate-1-^{14}C	Control	16,800	

TABLE XVII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	3,900	
Lactate-1-^{14}C	PTE-Treatment	3,670	Slight Change
Lactate-1-^{14}C	PTE-Treatment	3,800	
Lactate-1-^{14}C	Control	4,470	
Lactate-1-^{14}C	Control	4,480	
Lactate-1-^{14}C	Control	4,100	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	16,000	
Lactate-1-^{14}C	PTE-Treatment	19,200	Slight Change
Lactate-1-^{14}C	PTE-Treatment	17,500	
Lactate-1-^{14}C	Control	15,100	
Lactate-1-^{14}C	Control	14,000	
Lactate-1-^{14}C	Control	17,400	

TABLE XVIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	7,500	Slight Change
Lactate-1-^{14}C	PTE-Treatment	7,450	
Lactate-1-^{14}C	Control	6,400	
Lactate-1-^{14}C	Control	5,880	

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	23,300	Slight Change
Lactate-1-^{14}C	PTE-Treatment	26,600	
Lactate-1-^{14}C	Control	18,800	
Lactate-1-^{14}C	Control	20,500	

to the controls. (Table XIX).

Experiment 18: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Glucose-1-^{14}C

Six thyroparathyroidectomized rats were used in the experiment. Three of the rats were injected subcutaneously with parathyroid extract, and the other three received injections of vehicle. The rats received 162 U.S.P. units of parathyroid extract which caused an average increase in serum calcium of 3.1 mg% over that of the control animals.

One microcurie of the radioactive substrate with 4.0 ml of bone homogenate were present in each incubation flask. The incubation was performed for three hours at 37° C.

The diaphyses of the femora showed an average increase of 52% in the oxidation of the substrate by the bone homogenate obtained from the PTE-treated animals when compared to the oxidation detected in the preparations obtained from the control animals. The epiphyses demonstrated no significant average change. (Table XX).

Experiment 19 and 20: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Glucose-2-^{14}C

Six thyroparathyroidectomized rats were used in both experiments. Three of the animals were injected subcutaneously with 150 U.S.P. units of parathyroid extract, and three received

TABLE XIX

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-2-^{14}C	PTE-Treatment	3,900	
Lactate-2-^{14}C	PTE-Treatment	3,700	+ 62%
Lactate-2-^{14}C	PTE-Treatment	3,900	
Lactate-2-^{14}C	PTE-Treatment	4,100	
Lactate-2-^{14}C	Control	2,400	
Lactate-2-^{14}C	Control	2,600	
Lactate-2-^{14}C	Control	2,000	
Lactate-2-^{14}C	Control	2,700	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-2-^{14}C	PTE-Treatment	24,400	
Lactate-2-^{14}C	PTE-Treatment	26,600	No Change
Lactate-2-^{14}C	PTE-Treatment	33,700	
Lactate-2-^{14}C	PTE-Treatment	25,000	
Lactate-2-^{14}C	Control	28,300	
Lactate-2-^{14}C	Control	28,300	
Lactate-2-^{14}C	Control	28,600	
Lactate-2-^{14}C	Control	29,000	

TABLE XX

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF GLUCOSE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-1-^{14}C	PTE-Treatment	5,600	+ 52%
Glucose-1-^{14}C	PTE-Treatment	5,300	
Glucose-1-^{14}C	Control	3,400	
Glucose-1-^{14}C	Control	3,800	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-1-^{14}C	PTE-Treatment	19,800	No Change
Glucose-1-^{14}C	PTE-Treatment	20,300	
Glucose-1-^{14}C	Control	18,300	
Glucose-1-^{14}C	Control	18,500	

injections of vehicle. The rats which received the parathyroid extract showed an average increase in serum calcium level of 2.4 mg% in experiment 19 and 2.6 mg% in experiment 20.

One microcurie of radioactive substrate with 4.0 ml of bone homogenate were present in each incubation flask. The incubation in experiment 19 was carried out for three hours, and the one in experiment 20 was run for one and one-half hours. Both incubations were run at a temperature of 37° C.

In experiment 19, the diaphyses of the femora demonstrated an average stimulation of 68% in the oxidation of the substrate by bone homogenate prepared from the PTE-treated animals when compared to the control animals. The epiphyses of the femora showed no significant changes. (Table XXI).

In experiment 20, the diaphyses of the femora showed an increase of 53% in the oxidation of the substrate by the bone homogenate prepared from the PTE-treated animals when compared to controls. The oxidation in the epiphyses of the femora showed no significant change. (Table XXII).

The incubations in these two experiments were conducted for different periods of time to determine optimal incubation time. The differences were detectable in the one and one-half hour incubation and were almost of the same magnitude as those observed for a three hour incubation. However, the activity detected was about half of the activity detected in the three hour incubation. This means that after one and one-half hours

TABLE XXI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF GLUCOSE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS
(Incubation Time: 3.0 Hours)

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-2-^{14}C	PTE-Treatment	2,160	+ 68%
Glucose-2-^{14}C	PTE-Treatment	1,700	
Glucose-2-^{14}C	Control	1,200	
Glucose-2-^{14}C	Control	1,100	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-2-^{14}C	PTE-Treatment	16,000	No Change
Glucose-2-^{14}C	PTE-Treatment	15,200	
Glucose-2-^{14}C	Control	15,800	
Glucose-2-^{14}C	Control	16,200	

TABLE XXII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF GLUCOSE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS (Incubation Time: 1.5 Hours)

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-2-^{14}C	PTE-Treatment	760	+ 53%
Glucose-2-^{14}C	PTE-Treatment	750	
Glucose-2-^{14}C	Control	490	
Glucose-2-^{14}C	Control	500	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-2-^{14}C	PTE-Treatment	3,700	No Change
Glucose-2-^{14}C	PTE-Treatment	4,100	
Glucose-2-^{14}C	Control	3,900	
Glucose-2-^{14}C	Control	3,300	

of incubation the tissue preparation are still metabolizing and the differences can still be detected.

Experiment 21: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Glucose-6^{14}C

Six thyroparathyroidectomized rats were used in the experiment. Three of the animals were injected subcutaneously with parathyroid extract and three were given injections of vehicle. The rats which received 150 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 2.9 mg% over the controls.

One-half microcurie of the radioactive substrate with 4.0 ml of bone homogenate was present in each flask. The incubation was performed for one and one-half hours at 37° C.

The diaphysis of the femora showed an average increase of 67% in the oxidation of the substrate by the bone homogenate prepared from the PTE-treated animals when compared to controls. The epiphysis of the femora showed a very slight difference. (Table XXIII)

Bone homogenization subjects the tissue to a great deal of traumatic stress. As described before, the tissue is quite disrupted and the homogenate appears as a milky suspension which will settle out rapidly. No premixed fortification (usually containing high energy phosphate, cofactors, and organic acids) is added to the homogenate. It was noted that the differences

TABLE XXIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF GLUCOSE-6-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-6-^{14}C	PTE-Treatment	230	+ 67%
Glucose-6-^{14}C	PTE-Treatment	225	
Glucose-6-^{14}C	Control	142	
Glucose-6-^{14}C	Control	154	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Glucose-6-^{14}C	PTE-Treatment	910	No Change
Glucose-6-^{14}C	PTE-Treatment	980	
Glucose-6-^{14}C	Control	1,100	
Glucose-6-^{14}C	Control	1,140	

detected in the homogenate preparations were less than those differences (average change) detected in the fragment preparations, although in both instances the differences were in the same direction. For example, Table XIV shows a 40% increase in the production of labeled carbon dioxide from pyruvate-2-^{14}C by a bone homogenate of diaphysis. In comparable experiments with femur fragments (Table IX), an increase of 75% in the production of labeled carbon dioxide from pyruvate-2-^{14}C was noted. A similar pattern was observed in femur epiphysis. Table XIV indicates a 15% increase in the production of labeled carbon dioxide from pyruvate-2-^{14}C by homogenates of femur epiphysis. A comparable study with the use of bone fragments (Table IX) shows a stimulation of 42% in the bone preparation obtained from the PTE-treated animals.

The differences in the oxidation of Krebs Cycle acids detected in fragments (Tables III-VII) completely disappeared when the tissue was homogenized (Tables XI-XIII).

It has been noted that PTE increases the amount of dinucleotide, especially NADP, in bone tissue (60). Perhaps an increased amount of NADP is necessary for PTE to have a metabolic effect on bone. Tissue homogenization could cause a net loss of dinucleotide from cells to the incubation media.

In view of this finding, it was decided to fortify the homogenate of bone with NADP. The results of these experiments appear in the following section.

Experiment 22: The Incubation of Nicotinamide Adenine Dinucleotide Phosphate Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,5-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with parathyroid extract and two were injected with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 3.1 mg% over the control animals.

One-half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The homogenate contained 2.0 micromoles of NADP per ml of homogenate. The incubation was carried out for three hours at 37°C.

The diaphyses of the femora demonstrated an average decrease of 55% in the oxidation of radioactive substrate by bone homogenate obtained from PTE-treated animals when compared to the control animals. The epiphyses of the femora showed an average increase of 60% in the oxidation of the substrate by bone homogenate obtained from the PTE-treated animals when compared to the controls (Table XXIV).

The average change detected in the NADP fortified homogenate more nearly paralleled the average change detected in the bone fragment study (Table III). Non-fortified homogenate showed no differences at all (Table XI).

TABLE XXIV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	2,100	- 55%
Citrate-1,5-^{14}C	PTE-Treatment	2,400	
Citrate-1,5-^{14}C	Control	5,100	
Citrate-1,5-^{14}C	Control	4,900	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Citrate-1,5-^{14}C	PTE-Treatment	14,600	+ 60%
Citrate-1,5-^{14}C	PTE-Treatment	15,400	
Citrate-1,5-^{14}C	Control	9,000	
Citrate-1,5-^{14}C	Control	9,800	

Experiment 23: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Succinate-2,3-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with vehicle. The rats which received 162 U.S.P. units of parathyroid extract showed an increase in serum calcium of 2.8 mg%.

One microcurie of radioactive substrate, 4.0 ml of homogenate, and 2.0 micromoles of NADP per ml of incubation media were present in each flask. The incubation was carried out for three hours at 37° C.

The diaphyses of the femora showed an average decrease of 23% in the oxidation of the substrate by bone preparation obtained from the PTE-treated animals. The epiphyses of the femora demonstrated an average increase of 45% in the oxidation of the substrate by bone preparations obtained from PTE-treated animals (Table XXV).

Experiment 24: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated Animals with 2-Oxoglutarate-5-^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with parathyroid extract, and two were injected with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.1 mg%.

TABLE XXV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	3,500	
Succinate-2,3-^{14}C	PTE-Treatment	3,200	- 23%
Succinate-2,3-^{14}C	PTE-Treatment	3,600	
Succinate-2,3-^{14}C	PTE-Treatment	3,900	
Succinate-2,3-^{14}C	Control	5,600	
Succinate-2,3-^{14}C	Control	4,000	
Succinate-2,3-^{14}C	Control	4,300	
Succinate-2,3-^{14}C	Control	4,500	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Succinate-2,3-^{14}C	PTE-Treatment	7,900	
Succinate-2,3-^{14}C	PTE-Treatment	8,200	+ 45%
Succinate-2,3-^{14}C	PTE-Treatment	7,900	
Succinate-2,3-^{14}C	PTE-Treatment	8,600	
Succinate-2,3-^{14}C	Control	5,200	
Succinate-2,3-^{14}C	Control	5,600	
Succinate-2,3-^{14}C	Control	5,900	
Succinate-2,3-^{14}C	Control	5,200	

One-half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The homogenate contained 2.0 micromoles of NADP per ml of homogenate. The incubation was performed for three hours at 37° C.

The diaphyses of the femora showed an average decrease of 21% in the oxidation of radioactive substrate by bone homogenate obtained from the PTE-treated animals when compared to the control animals. The epiphyses of the femora showed no difference in the oxidation of the substrate. (Table XXVI).

Experiment 25: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Pyruvate-2^{14}C

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected with parathyroid extract, and two were injected with vehicle. The rats which received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.5 mg% over the level in the control animals.

One microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The homogenate contained 1.0 micromoles of NADP per ml of homogenate. The incubation was carried out at 37° C for three hours.

The diaphyses of the femora showed an average increase of 75% in the oxidation of substrate by the bone homogenate obtained from the PTE-treated animals when compared to the

TABLE XXVI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF 2-OXOGLUTARATE-5-^{14}C BY BONE HOMOGENATES OF DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
2-Oxoglutarate-5-^{14}C	PTE-Treatment	4,100	- 21%
2-Oxoglutarate-5-^{14}C	PTE-Treatment	5,300	
2-Oxoglutarate-5-^{14}C	Control	6,200	
2-Oxoglutarate-5-^{14}C	Control	5,700	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
2-Oxoglutarate-5-^{14}C	PTE-Treatment	29,000	No Change
2-Oxoglutarate-5-^{14}C	PTE-Treatment	27,000	
2-Oxoglutarate-5-^{14}C	Control	24,000	
2-Oxoglutarate-5-^{14}C	Control	30,000	

control animals. The epiphyses of the femora demonstrated an average increase of 40% in the oxidation of the radioactive substrate by bone homogenate obtained from the PTE-treated animals when compared to the control animals. (Table XXVII).

Experiment 26 and 27: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Lactate-1-^{14}C

Four thyroparathyroidectomized rats were used in both experiments 26 and 27. Two of the rats were injected subcutaneously with parathyroid extract, and two were injected with vehicle. The rats which received 150 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.7 mg% in experiment 26 and 2.8 mg% in experiment 27.

One-half microcurie of radioactive substrate and four mls of homogenate were present in each flask. The homogenate contained 1 micromole of NADP per ml in experiment 26 and 2 micromoles per ml in experiment 27. The incubation was carried out at 37° C for three hours in a Dubnoff-type metabolic shaker bath. (Tables XXVIII, XXIX).

No significant differences were detected in either experiment. The same is true for studies done with non-fortified bone homogenates (Table XVI) and bone fragment studies (Table X).

TABLE XXVII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	38,300	+ 75%
Pyruvate-2-^{14}C	PTE-Treatment	31,400	
Pyruvate-2-^{14}C	Control	19,400	
Pyruvate-2-^{14}C	Control	20,900	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Pyruvate-2-^{14}C	PTE-Treatment	84,000	+ 40%
Pyruvate-2-^{14}C	PTE-Treatment	80,000	
Pyruvate-2-^{14}C	Control	52,200	
Pyruvate-2-^{14}C	Control	65,000	

TABLE XXVIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	4,200	No Change
Lactate-1-^{14}C	PTE-Treatment	3,100	
Lactate-1-^{14}C	Control	4,200	
Lactate-1-^{14}C	Control	4,300	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	9,600	No Change
Lactate-1-^{14}C	PTE-Treatment	10,200	
Lactate-1-^{14}C	Control	8,600	
Lactate-1-^{14}C	Control	10,300	

TABLE XXIX

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LACTATE-1-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	4,000	No Change
Lactate-1-^{14}C	PTE-Treatment	3,600	
Lactate-1-^{14}C	Control	4,800	
Lactate-1-^{14}C	Control	4,100	

EPIPHYSIS Substrate	Experimental Condition	Activity (cpm)	Average Change
Lactate-1-^{14}C	PTE-Treatment	20,000	No Change
Lactate-1-^{14}C	PTE-Treatment	23,700	
Lactate-1-^{14}C	Control	22,000	
Lactate-1-^{14}C	Control	19,000	

CHAPTER IV

DISCUSSION AND CONCLUSIONS

It has been proposed that PTH regulation of oxidative pathways could be a mechanism for controlling demineralization of bone (10, 11, 16, 28, 32, 38, 41, 42, 56, 60, 62).

Yates et al. (62) varied the levels of endogenous PTH via peritoneal lavage. After sacrifice of the lavaged animal, the femurs were removed and seperated into metaphyses (trabecular bone) and diaphyses (compact bone) in preparation for incubation. By measuring citrate and lactate concentrations present in the media, it was demonstrated that citrate production was greater in the diaphysis and lactate production was greater in the metaphysis.

The work presented by Yates appeared to indicate that PTH had a dual effect on trabecular and compact bone. The PTH effects on citrate production are mainly seen in the diaphysis of femur. The most numerous cell type of the diaphysis is the osteocyte, although osteoblasts and osteoclasts are also present. The epiphyseal cell types consist of a mixture of mesenchyme cells, osteoblasts, osteoclasts, and osteocytes. In general, the structure of the epiphysis is less highly organized than that of the diaphysis.

This research consisted of studying the evolution of $^{14}CO_2$ from various radioactive substrates using both diaphysis and epiphysis of femur from PTE-treated and control animals.

Cumulative data of the differences in the labeled carbon dioxide produced from the incubations in which bone fragments were used are given in Table XXX. In general, a depression in the evolution of $^{14}CO_2$ was observed in the incubations of Krebs Cycle intermediates performed with compact bone fragments and an increase in the evolution of $^{14}CO_2$ was noted in similar incubations done with trabecular bone fragments.

A decreased oxidation of citrate in the diaphysis would result in an accumulation of citrate. This observation agrees in principle with that of Yates et al. These workers attributed the increase in citrate to an increase in production, but they did not rule out a decrease in oxidation as an alternative explanation. When other Krebs Cycle intermediates were used in this study a decreased evolution of $^{14}CO_2$ was noted, but it was not as marked as that seen with citrate.

An increase in the oxidation of Krebs Cycle intermediates was consistently observed when epiphyseal bone fragments were used. Krebs Cycle intermediates are metabolized to a greater extent to carbon dioxide or some other organic acid under the influence of PTE. Yates found that citrate did not accumulate in trabecular bone after two hours of incubation. However, an increase in lactate was noted.

Glycolytic cycle intermediates pyruvate-2-^{14}C, and lactate-1-^{14}C were also studied. Pyruvate oxidation was stimulated to a greater degree in the diaphysis than in the epiphysis.

TABLE XXX

CUMULATIVE DATA OF DIFFERENCES IN THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LABELED SUBSTRATES BY PREPARATIONS OF FRAGMENTS OF DIAPHYSIS AND EPIPHYSIS FROM PTE-TREATED AND CONTROL ANIMALS

Substrate	Flasks	Diaphysis	Epiphysis
Citrate-1,5-^{14}C	16	- 60%	+ 70%
Succinate-2,3-^{14}C	16	- 30%	+ 40%
2-Oxoglutarate-5-^{14}C	8	- 40%	+ 12%
Fumarate-1,4-^{14}C	8	- 25%	+ 85%
Pyruvate-2-^{14}C	12	+ 75%	+ 42%
Lactate-1-^{14}C	16	No Change	No Change

Under the influence of PTE, the diaphysis incurs reduced Krebs Cycle activity. Perhaps another metabolic pathway such as glycolysis in bone is stimulated as a result of this depression or as a direct result of PTE. The increase in pyruvate oxidation was less than in the diaphysis and is probably a direct result of PTE, since there is no reduction of Krebs Cycle activity in the epiphysis.

The oxidation of lactate-1-^{14}C was not affected by PTE-treatment in either the diaphysis or the epiphysis. Yates et al. could detect an accumulation of lactate in the epiphysis after 2 hours of incubation, but a much greater accumulation was noted after 6 hours of incubation. The incubations performed in this research were carried out for not more than three hours. This time period was sufficient to detect changes in the oxidation of all other substrates except lactate-1-^{14}C.

In the second phase of the experimental work, homogenates were made of the bone tissue under consideration. As seen in Table XXXI, the effect on the Krebs Cycle intermediates found in the study of bone fragments disappears when the tissue is homogenized. Pyruvate maintained the effect in the diaphysis but not the epiphysis. Lactate showed no effect in either bone tissue. Other studies with glycolytic intermediates show a stimulation of oxidation in the diaphysis, but show no effect in oxidation in the epiphysis.

It was noted that the addition of NADP to the homogenate

TABLE XXXI

CUMULATIVE DATA OF DIFFERENCES IN THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LABELED SUBSTRATES BY PREPARATIONS OF HOMOGENATES OF DIAPHYSIS AND EPIPHYSIS FROM PTE-TREATED AND CONTROL ANIMALS

Substrate	Flasks	Diaphysis	Epiphysis
Citrate-1,5-^{14}C	16	No Change	No Change
Succinate-2,3-^{14}C	16	No Change	No Change
Pyruvate-2-^{14}C	28	+ 45%	+ 20%
Lactate-1-^{14}C	30	No Change	No Change
Lactate-2-^{14}C	12	+ 62%	No Change
Glucose-1-^{14}C	8	+ 52%	No Change
Glucose-2-^{14}C	16	+ 60%	No Change
Glucose-6-^{14}C	8	+ 67%	No Change

(Table XXXII) restored the effect seen in the study of bone fragments. In the oxidation of Krebs Cycle intermediates the PTE effect is dependent on the presence of an adequate amount of NADP in the tissue preparation. The relationship of NADP to PTE effect has been the subject of recent study (28, 29, 60). Apparently homogenization of bone results in a net loss or decreased availability of NADP to the oxidative process. However, homogenization does not seem to affect the oxidation of pyruvate in the diaphysis (Table XXXI), although addition of NADP does increase the stimulation of the oxidation (Table XXXII). This data helps substantiate Van Reen's finding that NADP levels in bone are increased under the influence of PTE and the theory of Hekkelman that PTH somehow reduces the availability of NADP to the NADP dependent enzymes of the Krebs Cycle.

The data from this research is interperted to mean there exists in bone two different metabolic processes which are affected in different ways by parathyroid extract. In femur, one of the processes is located mainly in the diaphysis and the other in the epiphysis. Neither process is mutually excluded from the area of the other. It is postulated that the type and ratio of bone cells in the diaphysis and epiphysis may be associated with the predominance of any one type of metabolism in any one region.

The loss of the ability to detect the parathyroid extract effect in bone homogenates can be attributed to cellular

TABLE XXXII

CUMULATIVE DATA OF DIFFERENCES IN THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF LABELED SUBSTRATES BY PREPARATIONS OF NADP FORTIFIED HOMOGENATES OF DIAPHYSIS AND EPIPHYSIS FROM PTE-TREATED AND CONTROL ANIMALS

Substrate	Flasks	Diaphysis	Epiphysis
Citrate-1,5-^{14}C	8	- 55%	+ 60%
Succinate-2,3-^{14}C	12	- 23%	+ 45%
2-Oxoglutarate-5-^{14}C	8	- 21%	No Change
Pyruvate-2-^{14}C	8	+ 75%	+ 40%
Lactate-1-^{14}C	16	No Change	No Change

disruption caused by homogenization. The addition of nicotinamide adenine dinucleotide phosphate restores the effect of parathyroid extract which was demonstrated in bone fragments. Van Reen (60) has demonstrated nicotinamide adenine dinucleotide phosphate levels in bone are increased by parathyroid extract-treatment. Perhaps the need for elevated levels of nicotinamide adenine dinucleotide phosphate is prerequsite for any demonstratable effect of parathyroid extract on bone metabolism.

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Abstract of the thesis entitled "PARATHYROID EXTRACT-INDUCED ALTERATIONS IN THE OXIDATION OF ORGANIC ACIDS IN BONE" submitted by Allyn F. DeLong in partial fulfillment of the requirements for the degree of Master of Science, June 1966.

Fragments and homogenates of femur diaphysis and epiphysis from rats receiving parathyroid extract and from control animals were incubated with various carbon-14 labeled substrates. Bone fragments of femur diaphysis prepared from parathyroid extract-treated animals showed a depression in the oxidation of Krebs Cycle intermediates, as measured by labeled carbon dioxide evolution from the substrate. Bone preparations of epiphysis demonstrated an increase in oxidation of substrate of approximately the same magnitude. However, the oxidation of pyruvate was increased and that of lactate showed no effect in both bone preparations of diaphysis and epiphysis.

When homogenates of femur diaphysis and epiphysis were used, the parathyroid status of the animals had no influence on the oxidation of Krebs Cycle intermediates or lactate. A moderate increase in the oxidation of pyruvate was noted in homogenates of diaphysis obtained from parathyroid extract-treated animals. The increase seen in the oxidation in tissue homogenate was not as great as that seen in bone fragment tissue. Additional experiments using glucose demonstrated an increase in oxidation of substrate in the diaphysis but no effect in the epiphysis.

The addition of nicotinamide adenine dinucleotide phosphate to homogenates restored the parathyroid extract effect seen in bone fragments with Krebs Cycle intermediates and pyruvate.

The data is interpreted to mean there exists in bone two different metabolic processes which are affected in different ways by parathyroid extract. In femur, one of the processes is located mainly in the diaphysis and the other in the epiphysis. Neither process is mutually excluded from the area of the other. It is postulated that the type and ratio of bone cells in the diaphysis and epiphysis may be associated with the predominance of any one type of metabolism in any one region.

The loss of the ability to detect the parathyroid extract effect in bone homogenates can be attributed to cellular disruption caused by homogenization. The addition of nicotinamide adenine dinucleotide phosphate restores the effect of parathyroid extract which was demonstrated in bone fragments. Perhaps the need for elevated levels of nicotinamide adenine dinucleotide phosphate is prerequisite for any demonstratable effect of parathyroid extract on bone metabolism.

APPROVAL SHEET

The thesis submitted by Allyn F. DeLong has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 26, 1966
Date

Maurice V. D'Heureux
Signature of Advisor